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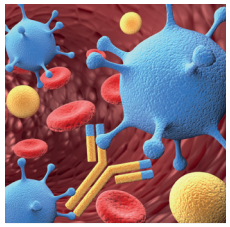
Surface Plasmon Resonance imaging (SPRi)

Streamlined SPRi-MS coupling to detect and identify a kinase in Cell lysate using the DARPins as binders



Application Note

Biology SPRi 41

Fatima-Ezzahra Hibti[1], Ulrike Anders[2] and Chiraz Frydman[1]
[1]HORIBA Scientific, Palaiseau, France; [2]ETH, Zurich, Switzerland

DARPins (Designed Ankyrin Repeat Proteins) are a class of non-immunoglobulin binders. Thanks to their specificity and robustness, they allow a multitude of novel, so far unfeasible applications. Surface Plasmon Resonance imaging (SPRi) is a powerful label-free technique that enables real-time target detection. Combining SPRi to mass-spectrometry (MS) allows biomolecules identification using their unique peptide mass fingerprint. In the past this combination was cumbersome and time-consuming. This application note shows how a protein kinase (RPS6KA2), a potential drug target is detected and identified using a hyphenated On-Chip SPR-MS coupling protocol, leading to saving time and money.

Key words: Surface Plasmon Resonance imaging, Mass spectrometry, MALDI-TOF, DARPins, protein kinase, cell lysate.

Introduction

Designed Ankyrin Repeat Proteins (DARPins) are multipurpose affinity reagents that can recognize targets with high specificity and affinity. They represent a very promising class of non-immunoglobulin binders that rival antibodies for target binding. Ribosomal protein S6 kinase alpha-2 (RPS6KA2) is an enzyme whose activity has been implicated in controlling cell growth and differentiation. It was identified as a presumed tumour suppressor in ovarian cancer[1].

SPRi (Surface Plasmon Resonance imaging) is a label-free and real time technique that's increasingly used for target detection and screening. One drawback of the SPRi is that the target cannot be identified. Combining SPRi and MALDi-Mass spectrometry (Matrix assisted laser desorption/ ionization) enables to overcome this limitation. The standard protocol (Off-Chip) is based on laborious sample elution and has several drawbacks like the dilution and loss of sample. For this study we choose designed ankyrin repeat proteins (DARPins) as binders[2] and we show how hyphenated On-Chip SPRi-MS protocol enabled us to detect and identify this potential drug target[3] in complex medium.

Materials and methods

DARPins immobilization using the Micropipette SPRi-Arrayer on a CS SPRi-Biochip

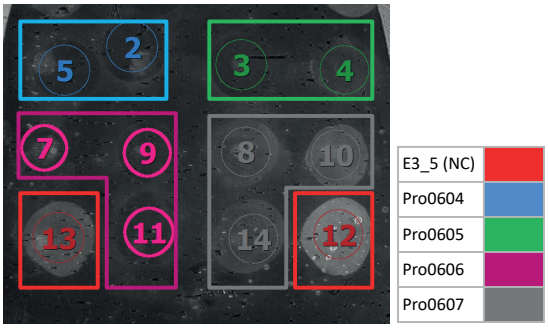
A CS SPRi-Biochip™ was used to immobilize the DARPins. The Micropipette SPRi-Arrayer uses contact spotting to immobilize biomolecules on the SPRi-Biochip surface in a 4x4 format. 0.3µL of a 0.1mg/mL protein solution (DARPins) were spotted on the chip. 10mM aqueous sodium acetate (pH5) was used as immobilization buffer.

The spots were kept in a humid atmosphere for 1h in a cooled ultrasonic cleaner and then washed with MilliQ water. For blocking the remaining free ester groups, the surface was immersed in 1M ethanolamine for 15min on a vibrating plate. Afterwards the slide was washed with water and a regeneration solution (100mM glycine in HCl, pH2) was added for an additional 15min on a vibrating plate to allow all slightly bound ligands to dissociate. Runs were performed for 12min, with 3min for the association and 9min for the dissociation step.

[1] Bignone PA. RPS6KA2, a putative tumour suppressor gene at 6q27 in sporadic epithelial ovarian cancer. Oncogene 2007 1; 26(5):683-700.
[2] Plückthun A. Designed ankyrin repeat proteins (DARPins): binding proteins for research, diagnostics, and therapy. Annu Rev Pharmacool Toxicol. 2015; 55 (1):489–511.
[3] Milosevic N. Synthetic lethality screen identifies RPS6KA2 as modifier of epidermal growth factor receptor activity in pancreatic cancer. Neoplasia. 2013; 15(12): 1354-62.

SPRi experimental details

The SPRi experiment was carried out using XelPleX™ system in 10mM PBS pH7.4. This high performance and fully-automated system is equipped with a continuous flow pump and an integrated autosampler fully controlled by the EzSuite software. The working temperature is regulated using a Peltier system. The flow rate was set to 50µL/min. 150µL of the protein was injected through the fluidic system. The purified kinase was injected first, then in the crude sample at increasing concentrations. To maximize the capture, five recycling cycles were added for each analyte injection. The surface of the sensor Biochip was regenerated using 100mM Glycine-HCl pH2.0 for the purified samples and 50mM NaOH for crude samples.



MALDI MS experimental details

The MALDI MS experiment was carried out using an Ultraflex II TOF (Bruker Daltonics, Bremen, Germany). In order to identify a protein from its peptide mass fingerprint, tryptic digests need to be performed. For this purpose, 0.3µL of a 5ng/µL trypsin gold solution in 10mM ammonium carbonate (pH8.3) was spotted on the region of interest and kept in a humid atmosphere at 37°C for 10min. To stop the digestion, the spots were dried. MALDI matrix (α-CHCA 7mg/mL in 50% acetonitrile/water with 0.3% TFA) was sprayed with an airbrush to deposit on the chip. The gold slide was mounted to an SPRi-MALDI adapter target and mass spectra were acquired in the positive ion reflectron mode. Each mass spectrum was the average of 2000 laser shots acquired at random sample positions. All mass spectra were smoothed, baseline-subtracted and externally calibrated with the standard peptide calibration mix I (LaserBio Labs). Tryptic peptides were identified with the Biotools software (Bruker Daltonics) and Mascot software (Matrix Science Limited).

Results and discussion

First, kinetic characterisation was monitored using the XelPleX system. Two conditions were tested: pure kinase in buffer solution and kinase in cell lysate.

1) Pure kinase in buffer solution results

Protein kinase was injected at 25nM, 50nM and 100nM with 5 recycling cycles for each injection. Specific binding was observed with both Pro0604 and Pro0606 DARPins (Figure 1).

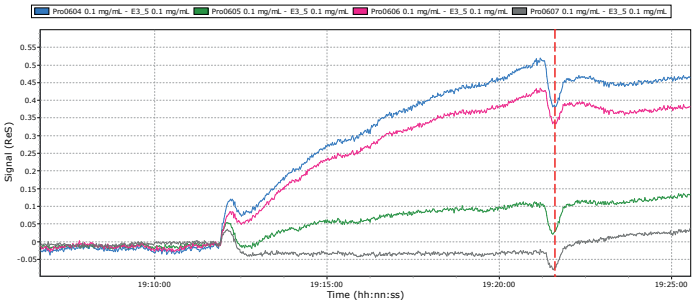


Figure 1: Kinetic curves after injection of RPS6KA2 protein at 100nM

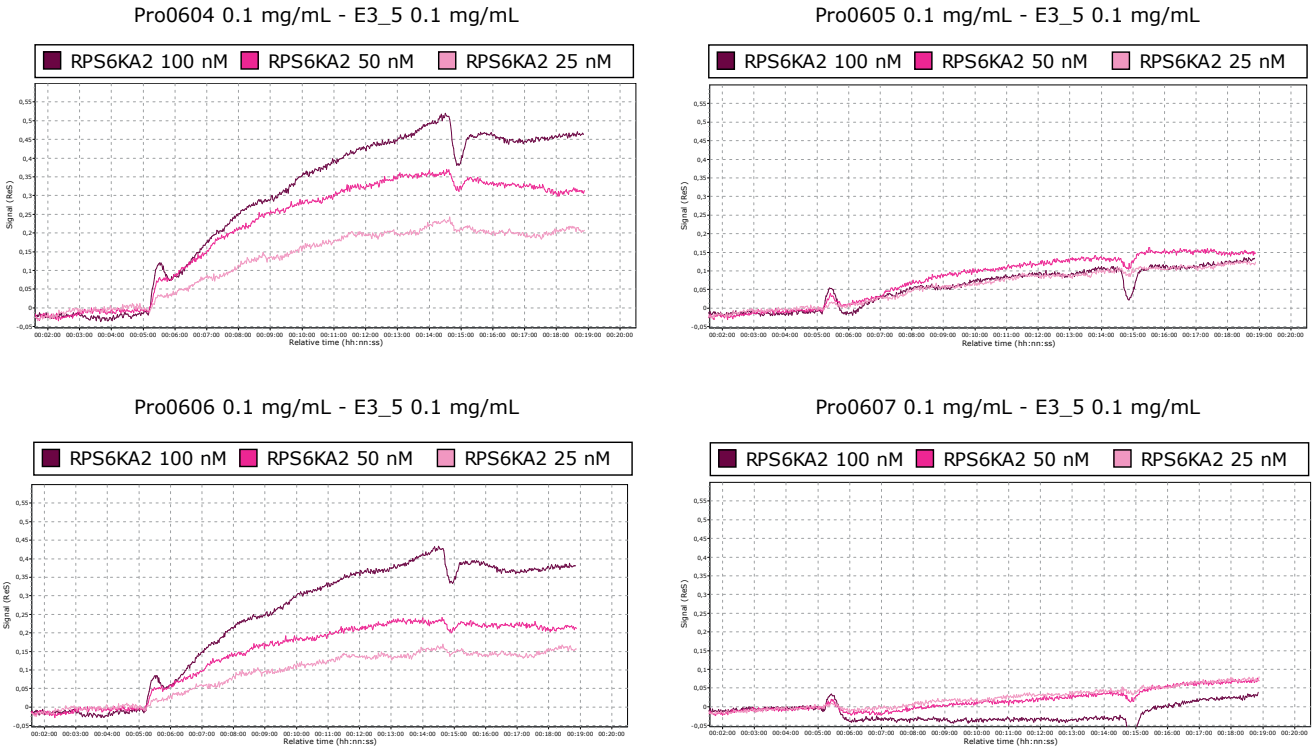


Figure 2: Averaged, reference-subtracted and superimposed Kinetic curves after injection of RPS6KA2 protein at 25, 50 and 100nM

Figure2 shows the superimposed injections for each immobilized DARPin.

The kinetic curves were fitted using EzFit to determine the kinetic constants. Figure 3 shows KD values of 20nM and 17nM for the DARPins Pro0604 and Pro0606 respectively.

2) Kinase in cell lysate

The cell extracts (SH-SY5Y) containing the protein kinase were also injected from 5nM to 160nM (estimated concentration of protein kinase). The surface was regenerated using 50mM NaOH after each crude sample injection.

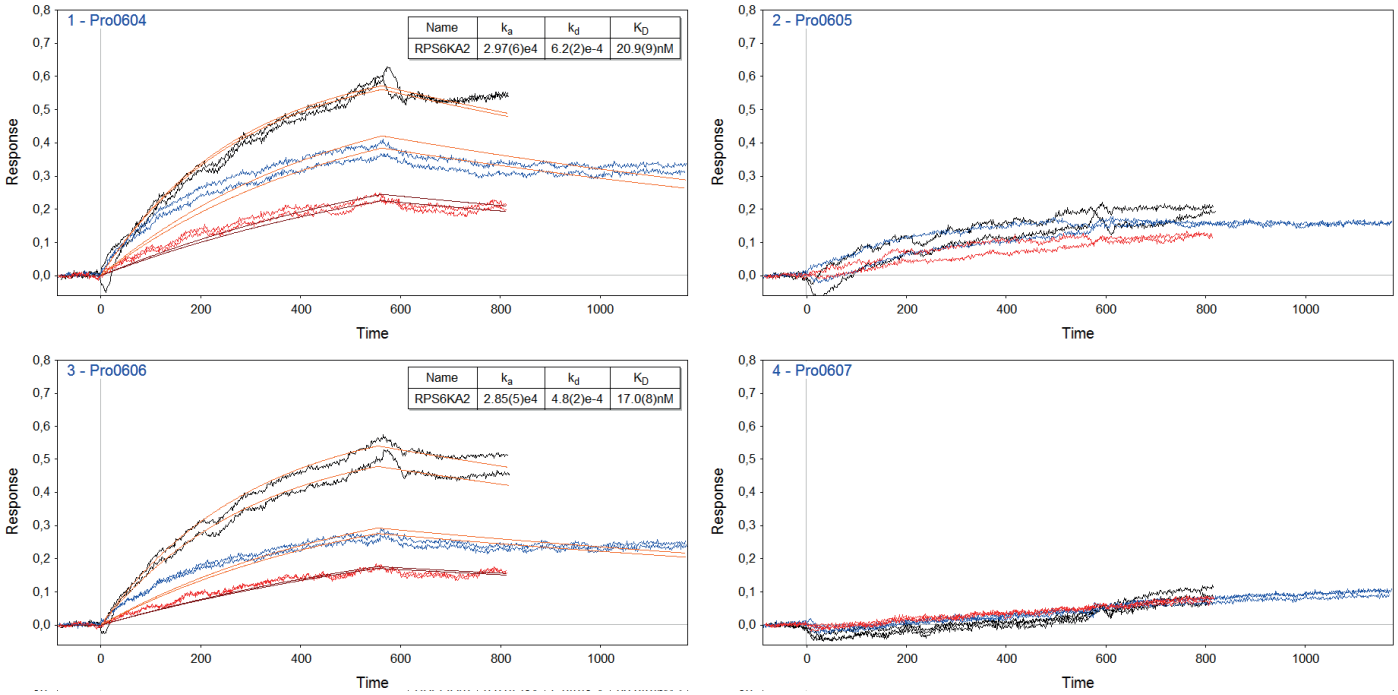


Figure 3: Fitted Kinetic curves after injection of RPS6KA2 protein at 25, 50 and 100nM

The table below summarizes the obtained KD.

	Pro0604	Pro0606
RPS6KA2	20.9nM	17.0nM

Figure 4 shows binding between the injected cell lysate and the DARPins Pro0604, Pro0605 and Pro0606. Thanks to the imaging, we can easily see the spots corresponding to the interacting DARPIns (Pro0604, Pro0605 and Pro0606) becoming white. In parallel, the spots of the negative control and Pro0607 still dark (Figure 5).

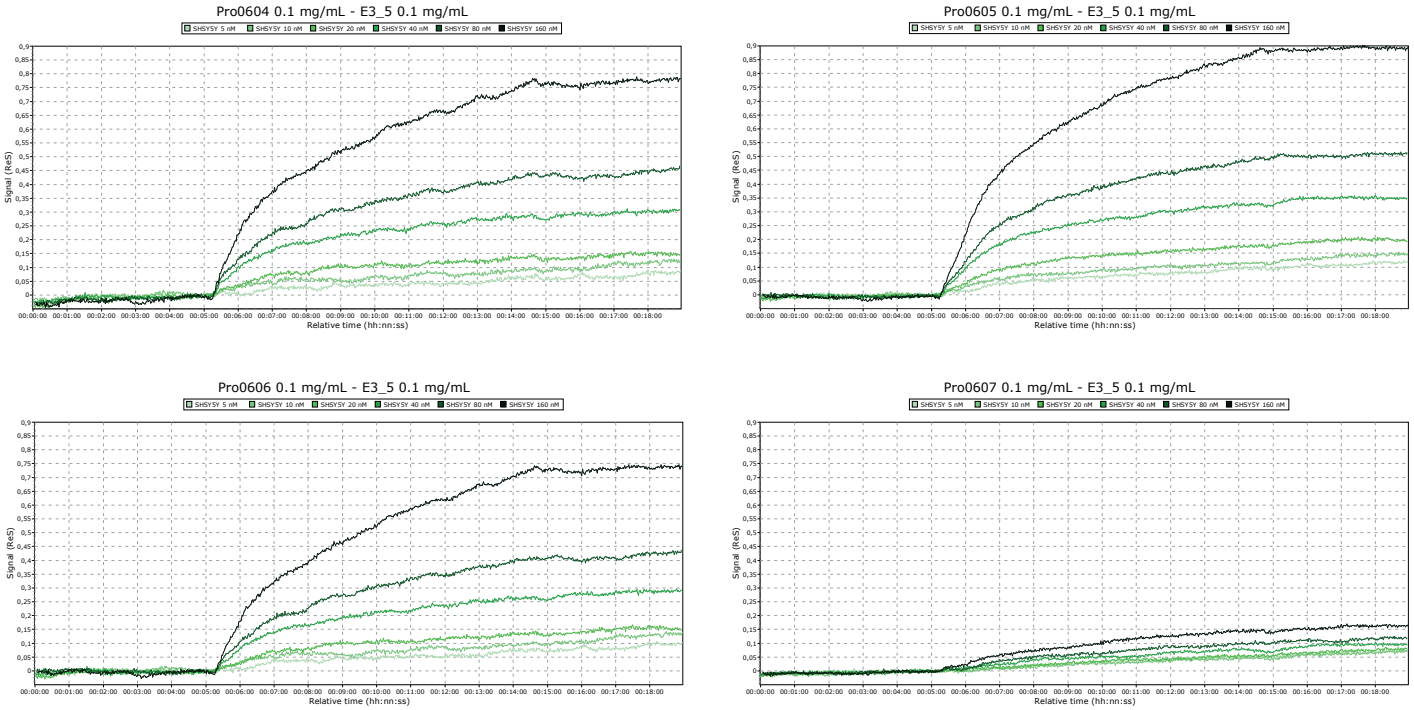


Figure 4 : Averaged, reference-subtracted and superimposed Kinetic curves after injection of SH-SY5Y cell extracts from 5nM to 160nM

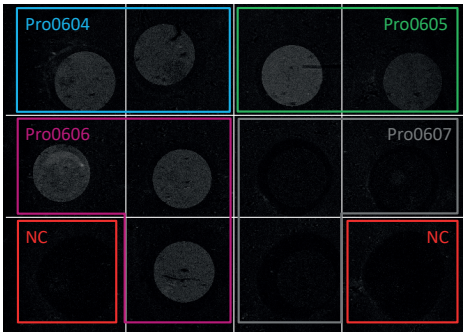


Figure 5 : Difference image after injection of SH-SY5Y cell extracts at 160nM

The fitted kinetics curves (Figure 6) point out affinity values around 800pM, 9,5nM and 1,4nM for Pro0604, Pro0606 and Pro0605 respectively.

The following table summarizes the evaluated KD for each DARPin.

	Pro0604	Pro0605	Pro0606
SHSY5Y	0.79nM	1.46nM	9.5nM

A second biochip was spotted within an array format using the same DARPins. Cell lysate sample was injected at 160nM with 35 recycling cycles, which enabled it to reach a contact time of ≈105min between the target (RPS6KA2) and the immobilized DARPins without regeneration (Figure 7).

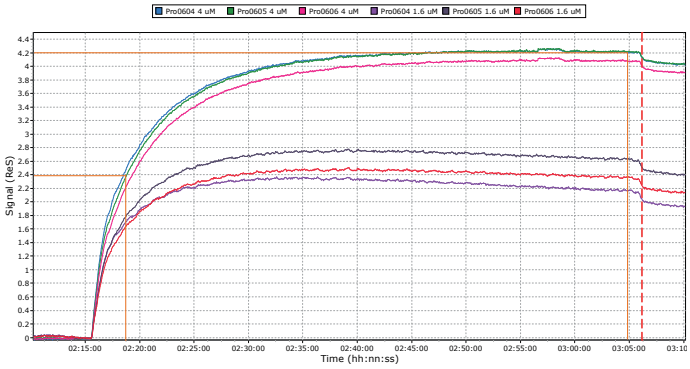


Figure 7 : Kinetic curves corresponding to the injection of cell lysates at 160nM with 35 recycling

Recycling the cell lysate over the sensor surface enabled an increase of the capture by 42%. The biochip was then removed from the XelPleX and prepared for MALDI MS analysis. In order to identify the target kinase by its peptide mass fingerprint, each spot was tryptically digested prior to MALDI matrix application. For digestion, an enzyme (e.g. trypsin) cleaves peptide bonds within a protein after specific amino acids (e.g. trypsin cleaves after arginine and lysine). These peptides form the so-called peptide mass fingerprint and support the assignment to a special protein, since every protein has its unique amino acid sequence and specific peptides are formed.

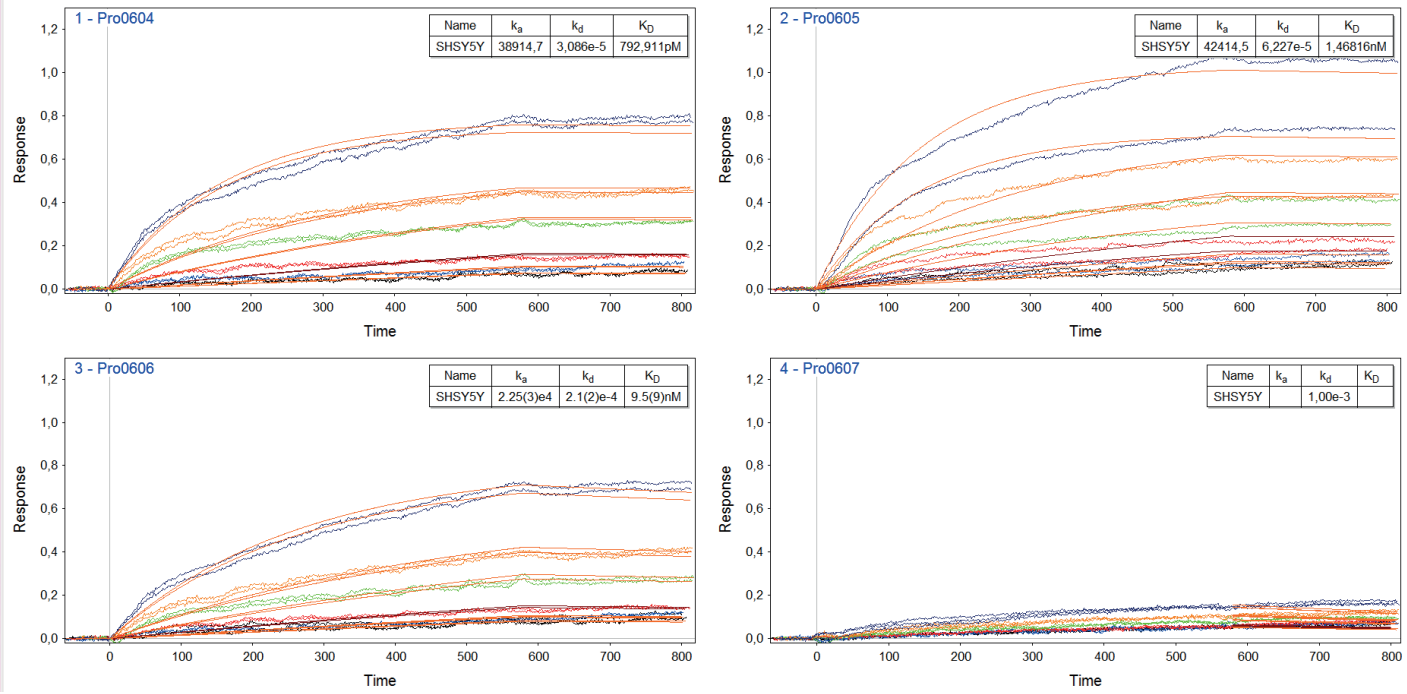


Figure 6 : Fitted Kinetic curves after injection of the cell extracts (SHSY5Y) from 5nM to 160nM

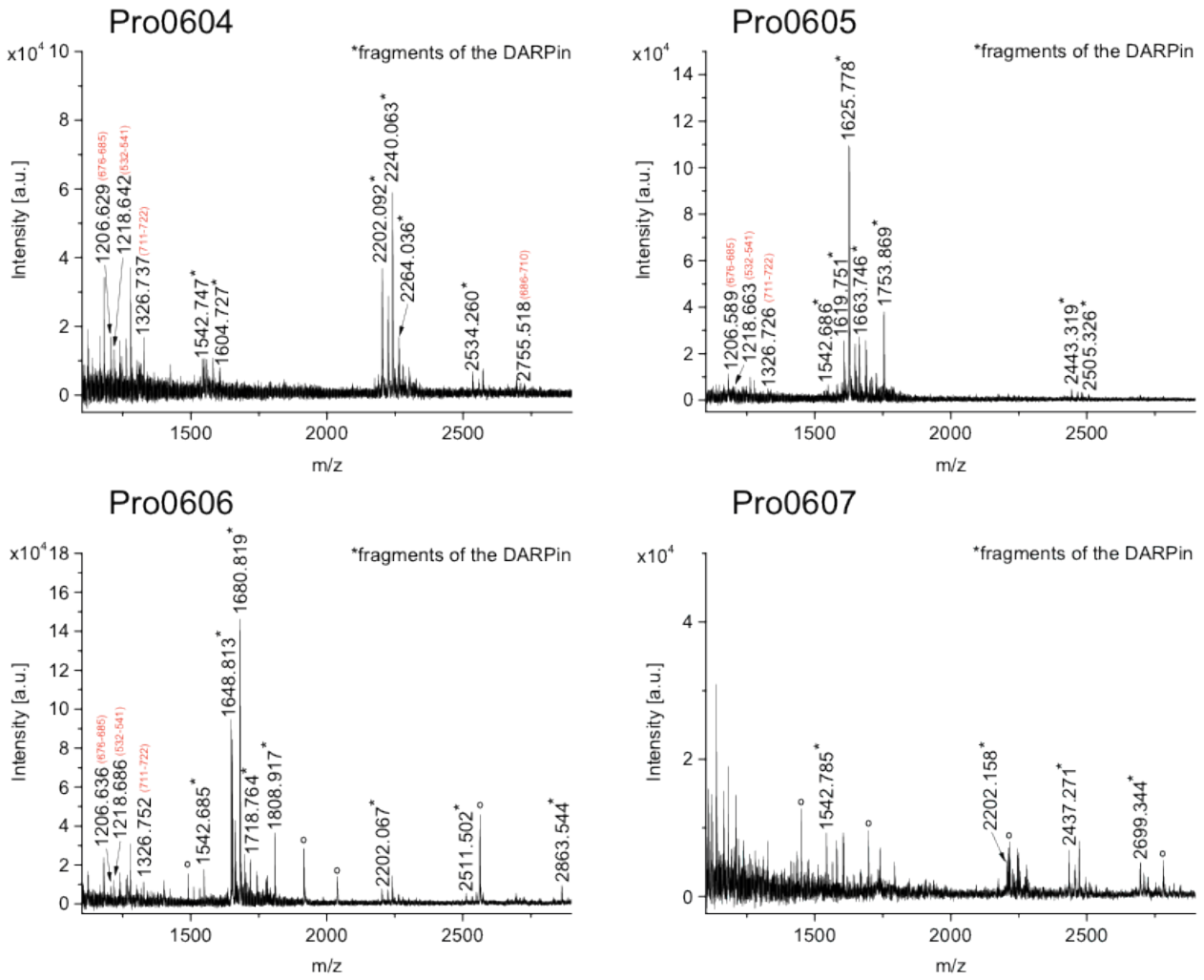


Figure 8: On-chip MALDI mass spectra after SPRi measurement and on-chip digestion of captured RPS6KA2 (from SH-SY5Y). Single charged ions were detected in positive ion mode. Fragments of RPS6KA2 are labelled with its sequence range. Peptides of the DARPins and tryptic autolysis peaks are marked with an asterisk or circle, respectively.

For the DARPins Pro0604, Pro0605 and Pro0606, peptides of RPS6KA2 could be identified (Figure 8, labelled with the amino acid sequence range), as there was an increase in the kinetic curve. A detailed look into the mass spectrum of Pro0604 shows four peptides of the target protein RPS6KA2 (with peptide ions at m/z = 1206.629, 1218.642, 1326.737 and 2755.518). These ions, which represent the peptide mass fingerprint of the targeted kinase, also allow for the clear identification of RPS6KA6. In addition, peptides of the DARPins themselves (since they are also proteins) were detected in each mass spectrum. Pro0607 showed no affinity in the cell lysate, which can be nicely seen in its mass spectrum. Only peptides of the DARPin or autolysis peptides from trypsin were detected. Furthermore, tryptic digested E3_5 used as a negative control also showed either peptides of the DARPin itself or trypsin autolysis peaks (not shown).

Conclusion

In this study, we demonstrated how a hyphenated and streamlined SPRi-MS coupling process enables the detection and the identification of a protein kinase in cell extracts. Thanks to its fluidic compatibility with the crude sample injections, the XelPleX enabled the detection of the kinase in cell lysate medium without any need for prior purification. In parallel, the recycling feature allowed maximum capture of the target prior to the mass spectrometry step. The protein kinase was clearly identified by its peptide mass fingerprint. This straightforward SPRi-MS coupling process dramatically changes the standard methods, which are money and time consuming. It leads the way to an easier kinetic and molecular characterization of pharmaceutical targets.